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**Evaluation Report on the Analytical Methods submitted
in connection with the Application for Authorisation of a
Feed Additive according to Regulation (EC) No 1831/2003**

**Preparation of *Bacillus paralicheniformis* DSM 33902 and
Bacillus subtilis DSM 33903 (Bovacillus™)
(FEED-2023-15650; CRL/230032)**



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in connection with the Application for Authorisation of a
Feed Additive according to Regulation (EC) No 1831/2003**

Dossier related to: **FEED-2023-15650 - CRL/230032**

Name of Product : ***Preparation of Bacillus paralicheniformis
DSM 33902 and Bacillus subtilis DSM
33903 (Bovacillus™)***

Active Agent (s): ***Bacillus paralicheniformis DSM 33902
Bacillus subtilis DSM 33903***

Rapporteur Laboratory: **European Union Reference Laboratory for
Feed Additives (EURL-FA)
JRC Geel, Belgium**

Report prepared by: **María José González de la Huebra**

Report checked by: **Zigmas Ezerskis**
Date: **17/11/2023**

Report approved by: **Christoph von Holst**
Date: **20/11/2023**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for a *preparation of Bacillus paralicheniformis* DSM 33902 and *Bacillus subtilis* DSM 33903 (*Bovacillus*TM) under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for the use of the *feed additive in compound feed and water* for all dairy ruminants.

According to the Applicant, the *feed additive* contains as active substances viable spores of non-genetically modified strains of *Bacillus paralicheniformis* DSM 33902 and *Bacillus subtilis* DSM 33903.

The *feed additive* is to be marketed as two solid formulations having a minimum total content of both microorganisms of 3.2×10^{10} Colony Forming Unit (CFU)/g. The *feed additive* is intended to be used directly in *compound feed* or into *water* at a minimum dose of 3.2×10^{10} CFU / kg and 7.4×10^7 CFU / L, respectively.

For the identification of *Bacillus paralicheniformis* DSM 33902 and *Bacillus subtilis* DSM 33903 at strain level, the EURL recommends for official control DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE) described in CEN Technical Specification (CEN/TS 17697).

For the enumeration of total *Bacilli spp.* (*Bacillus paralicheniformis* DSM 33902 and *Bacillus subtilis* DSM 33903) in the *feed additive, compound feed and water*, the EURL recommends for official control the ring-trial validated EN 15784 method.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Bacillus paralicheniformis DSM 33902 and *Bacillus subtilis* DSM 33903, *Bovacillus*TM zootechnical additives, gut flora stabilisers, other zootechnical additives, all dairy ruminants (including bovines, sheep, goat and buffalos).

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for a *preparation of Bacillus paralicheniformis* DSM 33902 and *Bacillus subtilis* DSM 33903 (*Bovacillus™*) under the category / functional group 4(b) 'zootechnical additives' / 'gut flora stabilisers', according to Annex I of Regulation (EC) No 1831/2003 [1]. The authorisation is sought for the use of the *feed additive* in *compound feed* and *water* for all dairy ruminants (including bovines, sheep, goat and buffalos) [2].

According to the Applicant, the *feed additive* contains as *active substances* viable spores of non-genetically modified strains of *Bacillus paralicheniformis* DSM 33902 and *Bacillus subtilis* DSM 33903 [3]. The strains are deposited at the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) under the deposit numbers DSM 33902 and DSM 33903, respectively [4].

The *feed additive* is to be marketed as two solid formulations *Bovacillus™* 10 and *Bovacillus™* WS, where the latter contains a different carrier for making it water-soluble form. Both formulations have a minimum content of total *active substances* of 3.2×10^{10} Colony Forming Unit (CFU) / g [3].

The *feed additive* is intended to be used directly in *compound feed* or into *water* at a minimum dose of 3.2×10^{10} CFU / kg *compound feed* and in *water* at a minimum dose of 7.4×10^7 CFU / L [5].

Note: The EURL previously evaluated the analytical methods for the determination of *Bacillus* spp. in the frame of several dossiers [6].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and the tasks of the European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with a *preparation of Bacillus paralicheniformis* DSM 33902 and *Bacillus subtilis* DSM 33903 and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, compound feed and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the enumeration of total *Bacilli spp.* (*Bacillus paralicheniformis* DSM 33902 and *Bacillus subtilis* DSM 33903) in the *feed additive, compound feed* and *water* the Applicant proposed [7] the ring-trial validated spread plate CEN method EN 15784 [8], which was recently revised by CEN. During the revision of the CEN method it was adjusted to VDLUFA method 28.2.2 [9] and completed with validation data from inter-laboratory studies using commercial feed products. The revision resulted in the updated CEN method dedicated for the enumeration of *bacilli spp.* in *feedingstuffs* (additives, *premixtures* and compound feeds including mineral feeds) that contain bacilli as a single microorganism component or in a mixture with other microorganisms [10].

Following the protocol of the updated CEN method, the sample (5 to 50 g) is suspended in 0.2 % sodium hydroxide solution containing Polysorbate 80 (Tween® 80) (tPBS). Decimal dilutions are prepared from the suspension using the above mentioned solution, spread plated on tryptone soya agar and incubated, aerobically at 37 °C for 16 to 24 h [10].

The following performance characteristics were reported from the ring-trial validation studies of non-transformed logarithmically CFU values of bacilli spp. ranging from 9.0×10^8 to 4.45×10^{14} / kg *feed additives, premixtures* and *compound feed* (including a mineral feed) [10]: a relative standard deviation for repeatability (RSD_r) ranging from 9.1 to 19.6 %; and a relative standard deviation for reproducibility (RSD_R) ranging from 17.1 to 33.9 %.

In addition, a limit of quantification (LOQ) of 3×10^7 CFU / kg can be calculated following the considerations of the ISO 7218 standard [11].

Furthermore, in the frame of the stability and homogeneity studies [12], the Applicant provided experimental evidences demonstrating the applicability of the above mentioned CEN method for the enumeration of the total Bacilli strains in the *feed additive, compound feed* and *water*.

Based on the performance characteristics and experimental data available, the EURL recommends for official control the ring-trial validated EN 15784 method for the enumeration of total *Bacilli spp.* (*Bacillus paralicheniformis* DSM 33902 and *Bacillus subtilis* DSM 33903) in the *feed additive, compound feed* and *water*.

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

An evaluation of corresponding methods of analysis is not relevant for the present application.

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008

For the identification of *Bacillus paralicheniformis* DSM 33902 and *Bacillus subtilis* DSM 33903 at strain level, the Applicant applied DNA sequencing methods such a comparative rRNA sequencing of the 16S region and Whole Genome Sequencing [13]. In former reports for similar dossiers, the EURL recommended for official control DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE), a generally recognised methodology for the genetic identification of bacterial strains. The method has been ring-trial validated and recently published as a CEN Technical Specification CEN/TS 17697 [14].

The EURL considers that both methodologies (PFGE and DNA sequencing methods, such as Whole Genome Sequencing - WGS) are suitable for official control for the bacterial identification of *Bacillus paralicheniformis* DSM 33902 and *Bacillus subtilis* DSM 33903 at a strain level.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation, the EURL recommends for the official control: (i) DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE) of CEN Technical Specification (CEN/TS 17697) for the identification of *Bacillus paralicheniformis* DSM 33902 and *Bacillus subtilis* DSM 33903; and (ii) the ring-trial validated spread-plate method EN 15784 for the enumeration of the total *Bacilli spp.* (*Bacillus paralicheniformis* DSM 33902 and *Bacillus subtilis* DSM 33903) in the *feed additive, compound feed* and *water*.

Recommended text for the register entry (analytical method)

- Identification: DNA sequencing methods or Pulsed-Field Gel Electrophoresis (PFGE) (CEN/TS 17697)
- Enumeration in the *feed additive, compound feed* and *water*: Spread-plate method on tryptone soya agar (EN 15784)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of a *preparation of Bacillus paralicheniformis* DSM 33902 and *Bacillus subtilis* DSM 33903 have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

- [1] Forwarding of applications for authorisation of feed additives in accordance with Regulation (EC) No 1831/2003 – E-Submission Food Chain platform:
<https://webgate.ec.europa.eu/esfc/#/applications/44850>
<https://open.efsa.europa.eu/questions/EFSA-Q-2023-00454>
- [2] *Application, Annex 1
- [3] *Technical dossier, Sect_II_Identity_Bov_1.ID+2.Charact_ConfMark
- [4] *Technical dossier, Sect_II_Annex II.2.1.2a
- [5] *Technical dossier, Sect_II_Identity_Bov_5.Cond_of_use_2023
- [6] EURL reports:
https://joint-research-centre.ec.europa.eu/feed-2021-0928_en
https://joint-research-centre.ec.europa.eu/finrep_fad-2021-0029-correlink.pdf
https://joint-research-centre.ec.europa.eu/finrep_fad-2021-0001_nsah007.pdf
https://finrep-fad-2020-0049-bacillus-amyloliquefaciens.docx_.pdf
https://joint-research-centre.ec.europa.eu/finrep_fad-2020-0058_bio-three.pdf
<https://joint-research-centre.ec.europa.eu/finrep-fad-2019-0090-mixbalac.pdf>
https://joint-research-centre.ec.europa.eu/finrep-fad-2019-0086_correlinkabs1781.pdf
https://joint-research-centre.ec.europa.eu/finrep-fad-2019-0074_correlink.pdf
https://joint-research-centre.ec.europa.eu/finrep_fad-2019-0044_syncra.pdf
<https://joint-research-centre.ec.europa.eu/finrep-fad-2019-0009-galliprofit.pdf>
<https://joint-research-centre.ec.europa.eu/finrep-fad-2018-0064-optimize.pdf>
https://joint-research-centre.ec.europa.eu/finrep-fad-2017-0058-baci_subtilis.pdf
- [7] *Technical dossier, Sect_II_Identity_Bov_6.Methods_2023
- [8] EN 15784:2009 - Animal feeding stuffs - Isolation and enumeration of presumptive *Bacillus* spp.
- [9] VDLUFA method – Enumeration of *Bacillus licheniformis* and *Bacillus subtilis* (VDLUFA Methodenbuch Bd.III, 28.2.2)
- [10] EN 15784:2021 - Animal feeding stuffs: Methods of sampling and analysis - Isolation and enumeration of *Bacillus* spp. used as feed additive
- [11] EN ISO 7218:2007 - Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations
- [12] *Technical dossier, Sect_II_Identity_Bov_4.Physio_chem_2023
- [13] *Technical dossier, Annex_II_2.1.2b_ID_certificates_Bov_Redacted
- [14] CEN/TS 17697:2023 - Animal feeding stuffs - Methods of sampling and analysis - PFGE typing of *Lactobacilli*, *Pediococci*, *Enterococci* and *Bacilli* in animal feeds

*Refers to Dossier no: FEED-2023-15650

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, Geel, Belgium. This report is in accordance with the opinion of the consortium of National Reference Laboratories as referred to in Article 6(2) of Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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